Confocal Microscopic Analysis of Integrin Expression on the Microvasculature and its Sprouts in the Neonatal Foreskin

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Members of the integrin family of adhesion receptors are essential participants in blood vessel growth and remodeling. It is not known which integrins are involved in the initial stages of angiogenesis *in vivo*. In this study we determined the location of integrins on the blood vessels of a growing tissue, the neonatal foreskin, in which neovascularization is likely to occur. We used the confocal microscope to visually reconstruct vessels from the papillary dermis of the foreskin and to identify potential sprouts as narrow, tapering extensions from these vessels. Blood vessels were initially identified by their positive reaction with antibodies to von Willebrand factor or human platelet endothelial cell adhesion molecule and their negative response to anti-neurofilament antibodies. Later, vessels were identified by their shape and location. We screened vessels with anti bodies to integrin subunits α1, α2, α3, α5, α6, αv, β1, β3 and β4. We found that integrin subunits α6 and β4 were consistently found along the whole length of capillary loops and extended to the distal ends of presumed sprouts. The α2 and αv integrin concentrations, which are normally low in the microvasculature, were increased on the sprouts. α5 was either absent from vessels entirely or more concentrated on the body than on the sprout. α1 was more commonly present on nerves than blood vessels. These studies suggest an important role for the α6β4 integrin in the initial stages of endothelial outmigration during new vessel growth. ***Key words: endothelial sprout/adhesion/angiogenesis/extracellular matrix. J Invest Dermatol 103:381–386, 1994***

Angiogenesis, whether during development or wound healing, entails the sprouting of new vessels from preexisting vessels. Endothelial cells extend processes through breaks in the basement membrane and then migrate as a column into the interstitial matrix. This migration consists of pairs of endothelial cells sliding past each other. Proximally the column may contain a lumen, but distally it is a solid cord [1–3].

Cell migration is a complex process requiring adhesion receptors to provide traction without binding cells too tightly to the substratum, and intracellular signaling to realign the cytoskeleton and initiate cortical flow [4]. Members of the integrin family of heterodimeric transmembrane receptors are involved in cell-substratum and cell-cell adhesion and also initiate various intracellular signals. The integrins bind to extracellular matrix molecules through their external domain, and may directly attach to cytoskeletal elements (talin and α-actinin) via their cytoplasmic domain. The ligand specificity and signaling ability of integrins are determined by varying the combination of α and β subunits that make up the heterodimers. There are twelve known α subunits and eight known β subunits. The integrin subunits α1, α2, α3, α5, α6, β1, β3, β4, and β5 have been detected on endothelial cells [5–9].

Angiogenesis obliges endothelial cells to leave the collagen IV/laminin-rich basement membrane and migrate through a matrix rich in interstitial collagen and fibronectin. We assumed that this change of substratum would require a different array of integrins on the endothelial sprout. Our purpose was to determine which integrins are important in the initial stages of blood vessel growth and repair. To do so, we have used confocal microscopy to reconstruct vessels from laser sections and identify sprouts as thin cords arising from vessels.

The confocal microscope, which allows the use of thick tissue sections (10 μ or more are visually sectioned down to 0.5–1.0 μ and then reconstructed by computer), has several advantageous features for examining sprouts. Because vessels can be studied over longer distances than usual, a) sprouts can be visualized, b) molecules can be compared by double-labeling immunofluorescence for their location along the sprout, and c) chances of "sprout" artifacts are greatly decreased because serial reconstruction shows whether a thin strand is truly a vessel outgrowth or only part of a longer vessel. In addition, the thick sections diminish the possibility that a vessel "end" will be created by cutting off the rest of the vessel.

We used double-labeling immunofluorescence microscopy to compare the location of various integrins on vessel sprouts with the location of the α6 integrin. The α6 integrin was chosen as a standard because antibodies to α6 produce intense staining of all endothelial cells at blood vessel basement membranes [9], and because we noted that this staining appeared to extend far into sprouts. The α6 integrin is also found on the epidermis and glands, and on infiltrating cells such as macrophages, but is not found on fibroblasts or smooth muscle cells by tissue staining [10]. Our studies indicate that integrins α6 and β4 are found on the narrow extensions of sprouts

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Abbreviation: PECAM, human platelet endothelial cell adhesion molecule.

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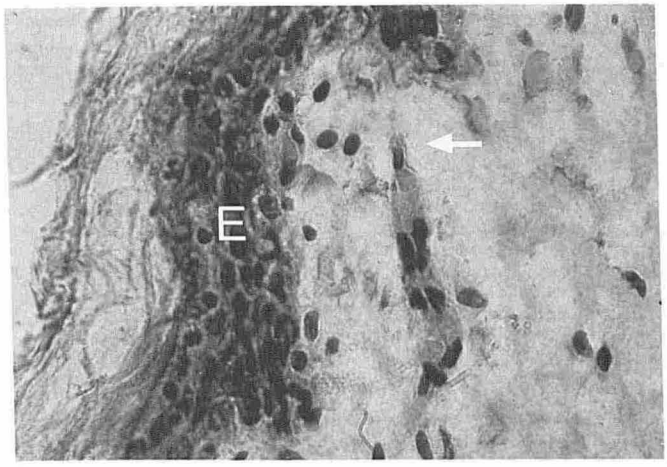

Figure 1. Hematoxylin and eosin stain of neonatal foreskin. *Arrow* indicates capillary tip approaching epidermis (E).

and may show a concentration at the distal tip. $\alpha 2$ and αv, though less concentrated than $\alpha 6$, are also found along the sprouts. Integrins $\alpha 5$ and $\beta 3$ are more prevalent on the body of blood vessels, and are entirely absent from some vessels.

MATERIALS AND METHODS

Tissue Preparation Human foreskin was obtained from the Well-baby Nursery at the University of California San Francisco. Tissues were fixed for 4 h in 4% formaldehyde in phosphate-buffered saline (PBS), transferred to 15% sucrose for 1 h, embedded in O.C.T. (Miles Laboratories, Elkhart Lake, Indiana), and frozen in liquid nitrogen. Tissue blocks were stored at −80°C. Frozen sections 10–18 μ thick were placed on superfrost glass slides (Fisher).

Immunofluorescent Staining All incubations and washes were done at room temperature in 1% goat serum in PBS. Nonspecific staining in tissue sections was blocked by incubating for 1 h in 1% goat serum in PBS. Sections were then treated with primary antibodies for 20 min, rinsed five times, treated with secondary antibodies conjugated to fluorescein (at 1:50) or rhodamine-lissamine (at 1:1000) for 20 min, washed five times, and mounted in Fluoromount-G (Fisher Biotech, Pittsburgh, PA). In all cases shown, $\alpha 6$ was labeled with rhodamine.

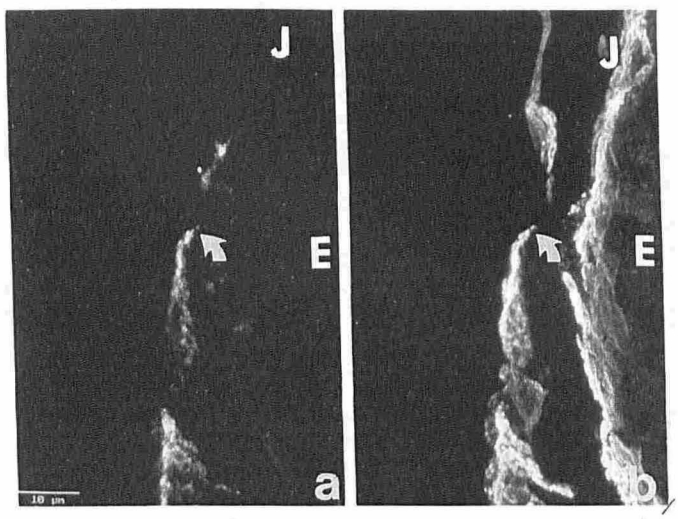

Figure 2. Expression of $\alpha 6$ on PECAM-positive vessels. Projection of confocal Z series. Neonatal foreskin was simultaneously labeled by indirect immunofluorescence with antibodies to a) the endothelial cell marker PECAM (with fluorescein secondary) and b) the $\alpha 6$ integrin (with rhodamine secondary). $\alpha 6$ is found both on blood vessels and on the dermal-epidermal junction (J). Note narrowing of vessel near tip *(arrows)*. E, epidermis. *Bar,* 10 μm.

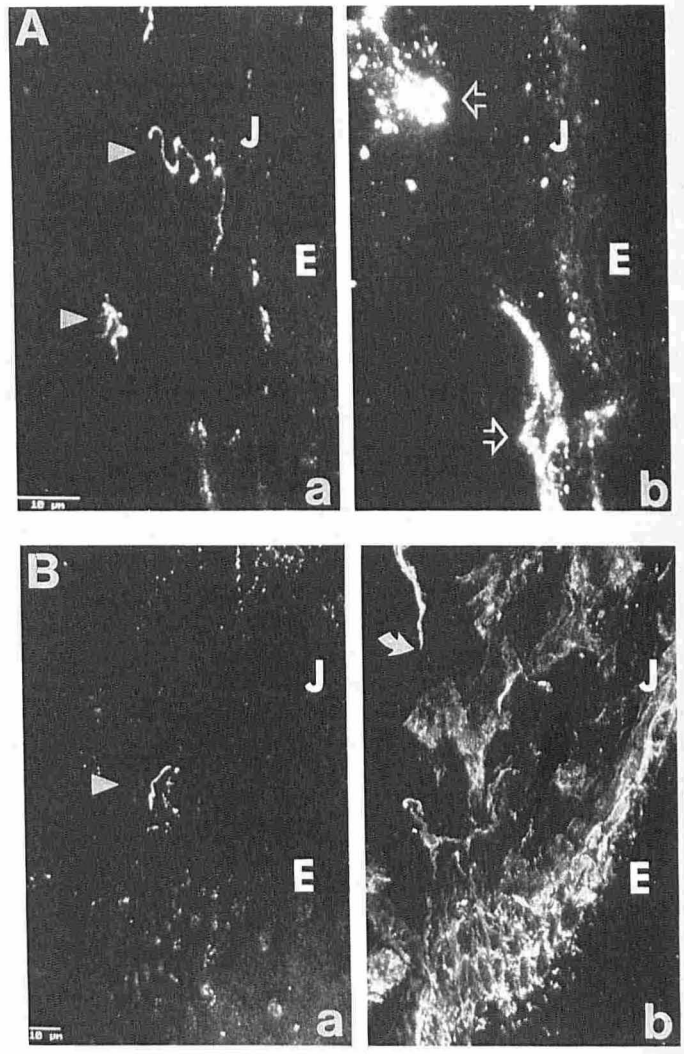

Figure 3. Neurofilament localization in foreskin. Projection of confocal Z series. *A)* The shape of fluorescein-labeled neurofilaments (a) is distinct from that of rhodamine-labeled vWF-positive blood vessels (b). *Arrowheads,* nerves. *Hollow arrows,* blood vessels. *B)* Fluorescein-labeled neurofilaments (a) are distinct from rhodamine-labeled $\alpha 6$ extensions (b). *Arrow,* tip of vessel extension. E, epidermis. J, dermal-epidermal junction. *Bar,* 10 μm.

Antibodies Mouse anti-human $\alpha 1$ was from T-Cell Sciences, Cambridge, MA. Mouse monoclonal anti-$\alpha 2$ (12F1) was provided by Dr. Virgil Wood, University of California, San Diego, CA [11]. Mouse monoclonal anti-$\alpha 3$ (VM-2) was provided by Dr. Vera Morhenn, SRI International, Menlo Park, CA [12]. Rabbit antibody to $\alpha 5$ was provided by R. Hynes, MIT, Boston [13]. Rat monoclonal anti-$\alpha 6$ (J1B5) was provided by Dr. Caroline Damsky, UCSF, San Francisco, CA [14]. Mouse monoclonal anti-human integrin αv subunit (LM142) was provided by Dr. David Cheresh, Research Institute of Scripps Clinic, La Jolla, CA [15]. Mouse monoclonal anti-human integrin $\beta 1$ (A-1A5) was provided by Dr. Martin Hemler, Dana Farber Cancer Institute, Boston, MA [16]. Rabbit antibody to human β (anti-GpIIb-IIIa) was provided by Dr. David Phillips, UCSF [17]. Mouse monoclonal anti-$\beta 4$ (3E1) and polyclonal anti-human laminin were purchased from Telios Pharmaceuticals, San Diego, CA. Mouse monoclonal anti-epiligrin (GB3) was purchased from Accurate Chemical and Scientific Corporation, Westbury, NY. Polyclonal antibodies to von Willebrand factor (vWF) were purchased from Sigma Chemical, St. Louis, MO. Monoclonal antibodies to neurofilaments (200 kDa protein) were from Boehringer Mannheim, Indianapolis, IN. Monoclonal antibodies to human platelet endothelial cell adhesion molecule (PECAM) were from R and D Systems, Minneapolis, MN. Goat anti-mouse, anti-rat and anti-rabbit IgG conjugated with fluorescein-isothiocyanate and goat anti-mouse, anti-rat, and anti-rabbit IgG conjugated with lissamine-rhodamine were from Jackson Immuno-Research, West Grove, PA. Antibodies were tested to ensure that there was

no crossreactivity of anti-mouse secondary antibody with rat primary antibody or vice versa.

Microscopy Samples were viewed on a Biorad MRC 600 confocal microscope and analyzed with the COMOS program. The papillary dermis was searched for potential vessel sprouts (areas of vessel narrowing). These areas were visually sectioned to 1 μ by the microscope. The dermal-epidermal junction was used as a marker for determining tissue thickness. Sections were reconstructed into two-dimensional projections from a 1-μ series. All micrographs are projections of a Z series unless otherwise indicated.

RESULTS

We chose human neonatal foreskin in which to study microvascular development because there is significant neovascularization in skin during the first three postnatal months [18–20], and because a large number of probes are available for human integrins and their ligands. After birth, the disorganized subpapillary plexus in the skin is remodeled into discrete pathways, and capillary loops are projected deep into the dermal papilla. **Figure 1** shows a hematoxylin-and-eosin–stained section of neonatal foreskin. Note how the capillary tapers as it extends towards the epidermis.

We used both immunologic and morphologic criteria to identify sprouts arising from the microvasculature. First, blood vessels were identified within the dermis of human foreskin by their reaction with antibodies to the endothelial cell markers vWF or PECAM. Sprouts were recognized as tapering continuous extensions from these vessels that were verified not to be fragmented vessels by confocal sectioning. Whereas capillaries are typically 5–10 μm in diameter, the sprout tips are solid cords that narrow to ~2 μm or sometimes have wider club-like shapes [2]. The confocal microscope allows the observer to follow vessels through a thick tissue block and to distinguish the top and bottom of sections of the block. We consider that those extensions that taper and end in the middle sections of a series are likely to be sprouts and not cutting artifacts.

Our initial studies compared the location of various integrins with that of the blood vessel markers PECAM and vWF. **Figure 2** is a confocal two-dimensional reconstruction of neonatal foreskin double-labeled for PECAM and α6 by indirect immunofluorescence. The blood vessel tapers until it is too narrow to contain a lumen. PECAM colocalizes with α6 on both the vessel and the tapering extension. However, the dermal-epidermal junction is α6-positive and PECAM-negative. Comparison of other integrins with PECAM or vWF confirmed previous reports [5,9,21] that α2, α3, α5, αv, β1 and β4 are present on the body of some blood vessels of the microvasculature (not shown).

Because α6 stains blood vessels continuously and intensely, we chose α6 as the standard with which to compare the location of other integrins. However, because α6 is also found on nerves, we first compared the location and appearance of nerves, as labeled by antibodies to the 200-kDa neurofilament protein, with that of vWF or α6 in the papillary dermis **(Fig 3)**. Unlike the blood vessels, which taper into sprouts, the nerves remain very narrow over long distances. Sometimes the nerve fiber twists around a vessel or runs alongside a blood vessel. Figure 3*B* shows a tapering α6-positive vessel that does not react with anti-neurofilament antibodies.

Comparison of other integrins with α6 revealed that β4 colocalized on sprouts with α6 **(Fig 4)**. Figure 4*B* shows part of a Z series of the same tissue block. Note that the narrow vessel extension is longest in section 5 and gradually diminishes in length in subsequent sections. Like α6, antibodies to β4 stained basement membranes of endothelium and epithelium intensely. Whereas α6 and β4 were present along most of the vessel length, staining for both α2 and αv was pale on much of the vessel body, but was sometimes concentrated in the distal part of the vessel containing the sprout **(Fig 5*A,B*)**. The diffuse staining of α2 and αv suggested cytoplasmic localization rather than the basement membrane localization of α6. Antibody to α2 usually stained the basal epidermal cells much more intensely than the blood vessels **(Fig 5*A*)** whereas αv staining was patchy or faint in the epidermis **(Fig 5*B*)**. Antibody to β1, like that to α6, stained the full length of many vessels, but the two stains did not always colocalize either along the vessel or at the tip **(Fig 5*C*)**. Areas in which β1 and α6 did not colocalize likely represented $\alpha6\beta$4-rich sites. It was also noted that staining of the epidermis with anti-β1 antibodies produced a circumcellular pattern, without a distinct basement membrane stain, suggesting a lack of $\alpha6\beta1$ on the basement membrane. Anti-β3 stained some vessel bodies brightly **(Fig 5*D*)** and others not at all; it also extended along some sprouts (not shown). Three integrin subunits were almost never seen on vessel sprouts. Staining for the α5 integrin was either more concentrated on the vessel body than on tapering extensions or missing entirely **(Fig 5*E*)**. α5 was only infrequently seen on epidermal patches. Staining for α3, which was pale on blood vessels, was

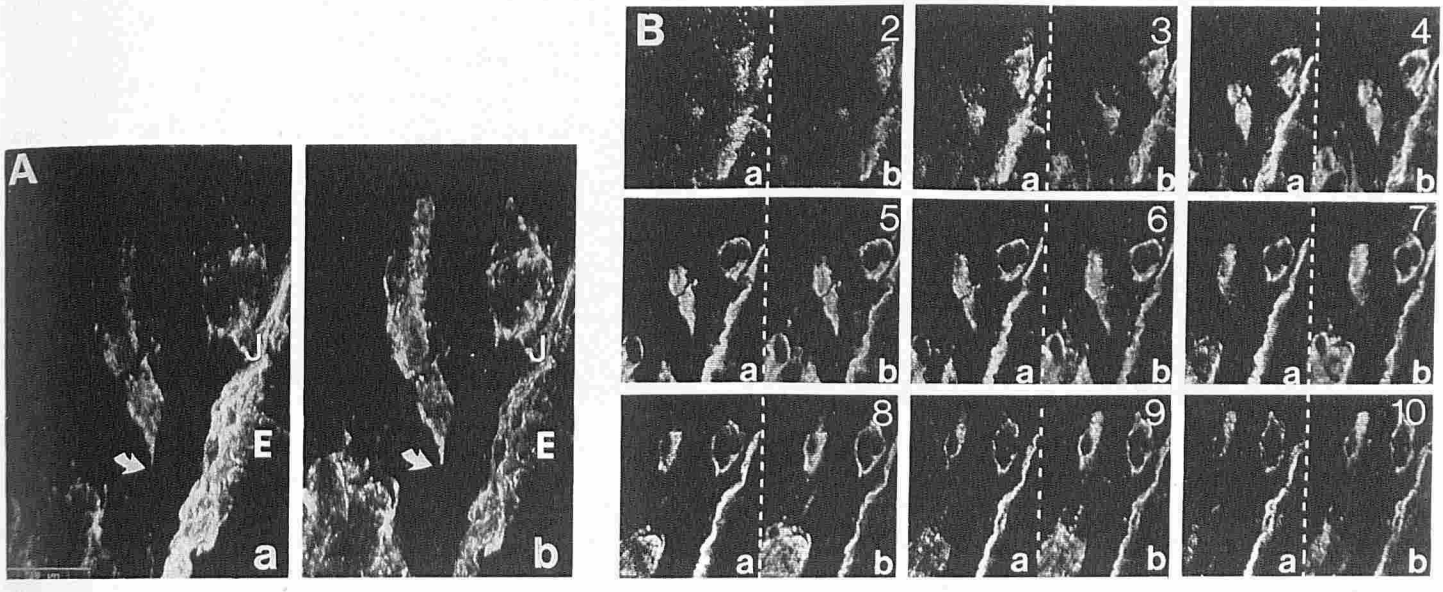

Figure 4. Colocalization of β4 and α6 in projection of Z series and individual Z-series sections. *A)* Projection of Z series. Fluorescein-labeled β4 (a) colocalizes with rhodamine-labeled α6 (b). *Arrow*, tip of vessel extension. E, epidermis. J, dermal-epidermal junction. *B)* 1 μ sections from the Z series. Sections 2–10 of a 12-section series are shown. Section 5 corresponds to the longest tapering extensions shown in *A*. *Bar*, 10 μm.

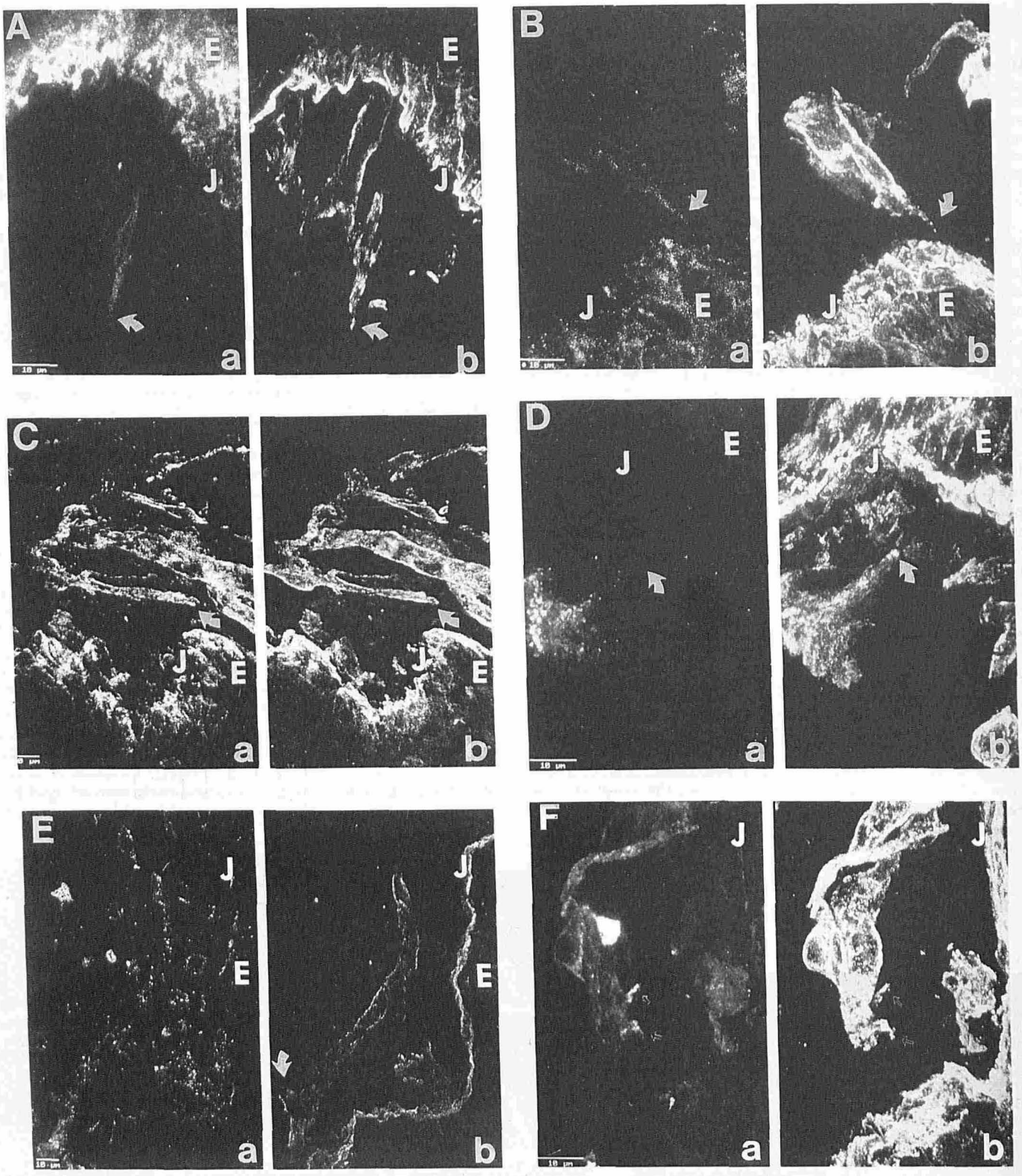

Figure 5. Localization of α2, αv, β1, β3, α5, and α1 on blood vessels. Projection of confocal Z series. Foreskin was double-labeled with fluorescein for either α2, αv, β1, β3, α5, or α1 and with rhodamine for α6. *A)* α2 is prominent in basal epidermal cells and diffusely present on the end of blood vessel extensions (a) as compared with α6 (b). *B)* αv is diffusely present near the vessel tip (a) as compared with α6 (b). Note the break in staining just before the tip on the α6-positive sprout. This break is characteristic of α6 in sprouts. *C)* β1 is brightly stained along the vessel length (a) but is not always colocalized with α6 (b). *D)* β3 is present on the blood vessel body (a) lesser amounts on the vessel extension, visualized by staining α6 (b). *E)* α5 is sometimes found along blood vessels (a) but is less distinct on vessel extensions, as visualized by staining α6 (b). *F)* α1 is predominantly found on the nerve (a) encircling the α6-positive vessel (b). Epidermis in *F* is on the right side of picture. E, epidermis. J, dermal-epidermal junction. *Arrows,* tips of vessel extensions. *Bar,* 10 μm.

concentrated on basal epidermal cells (not shown). α1 was more prevalent on nerves than on blood vessels **(Fig 5*F*)**.

We also compared the localization of α6 with that of its potential ligands: laminin, and the laminin variant epiligrin. Epiligrin was usually not present on blood vessels. A-chain laminin was present on all α6-positive vessels, as were its individual A and B subunits (data not shown).

DISCUSSION

Using the confocal microscope to reconstruct blood vessels, we have examined the localization of various integrins along the vessels and their newly forming sprouts. The integrins α6 and β4 were most consistently found on both the body of microvascular vessels and on the long, tapering sprouts. Integrins α2 and αv sometimes colocalized with the tips of these extensions, whereas α5, when present, was denser on the vessel body than on the distal end of the sprout.

The known ligands for α6β1 and α6β4 are members of the laminin family. The consistent localization of α6 and β4 at the distal ends of sprouts came as a surprise, because endothelial cells leave the laminin/type IV collagen-rich blood vessel basement membrane during angiogenesis and migrate through a matrix rich in fibronectin, fibrin, and interstitial collagen. However, studies of induced angiogenesis in corneas have detected laminin at the tips of newly forming vessels, and even on individual cells in advance of the tip [22,23]. When human microvascular endothelial cells are plated on fibronectin *in vitro,* they synthesize laminin within 24 h [23]. In addition, the endothelial cell line EAHY 926 forms tubes in the laminin-rich matrix Matrigel. Significantly, this tube formation is blocked by antibodies to α6 or β1, but not by antibodies to α5. Blockage occurs when as few as 10% of α6 or β1 sites are bound by antibody [24]. Much higher antibody concentrations are needed to block cell adhesion. Drake *et al* [25] provided evidence that an earlier stage of angiogenesis, cord formation, is not dependent on β1 integrins. They showed that when chick embryos are treated with the CSAT antibody, which perturbs β1 integrins, formation of blood vessel tubes from solid cords is blocked, but initial cord formation is unaffected. Therefore, if integrins function in cord formation *in vivo,* one would expect that a major component would be some non-β1 integrin, such as β4.

α6β4 is primarily associated with epiligrin in hemidesmosome complexes on epithelial cells [26]. However, well-developed hemidesmosomes are not present in the microvasculature. Because epiligrin (A. Sonnenberg, personal communication) and A-chain laminin [27] are ligands of α6β4 in other cell types, we attempted to stain for epiligrin with GB3 antibodies. Results were usually negative. Either epiligrin is not present or microvascular epiligrin is not accessible to the GB3 antibodies. A third possibility is that a variant epiligrin-like laminin isoform exists that could bind α6β4. In fact, a truncated variant of laminin has been detected in aortic endothelial cells [28]. We have observed that dermal microvascular endothelial cells do not make significant A-chain laminin, but seem to synthesize a truncated form of laminin (unpublished observations).

Although α6 and β4 were present along the whole length of the vessel and sprout, α2 and αv staining was relatively weak along the vessel body and was sometimes detectable only on the distal end of the vessel, including the sprout. The increased density of α2 and αv on vessel sprouts is evidence that they are active in angiogenesis. Previous *in vivo* studies have shown that both α2 and αv are present during capillary growth. α2 levels are high during embryonic glomerular capillary formation but low in capillaries in adults [29], and αvβ3 has been detected in human granulation tissue [21]. Concentrations of α2 and αv, as well as α5, are upregulated by the angiogenic factor basic fibroblast growth factor (bFGF) [30,31]. *In vitro* tube formation studies provide complex information on the role of α2 and αv. Human umbilical vein endothelial cells (HUVEC) plated on Matrigel were inhibited from forming tubes in the presence of anti-αv antibodies [32], whereas HUVEC plated on fibrin were stimulated to produce more and longer tubes in the presence of antibodies to αvβ3 and phorbol myristic acid [33]. Likewise, HUVEC plated on collagen were stimulated by antibodies to α2. However, αv and α2 antibodies were not stimulatory when the substrates were reversed (HUVEC were plated on collagen and fibrin, respectively). Thus, *in vitro* tube studies cannot yet predict which integrins are important during *in vivo* angiogenesis, a process that depends on a complex and changing mixture of ligands.

The finding that α5 and β3 are present only on a subset of microvascular endothelia is consistent with previous studies in which α5β1 and αvβ3 were present in granulation tissue but not quiescent blood vessels [21]. This in turn provides further evidence that the microvasculature in the neonatal foreskin is undergoing active remodeling. The presence of the fibronectin receptor, α5β1, on wider parts of vessels but not the narrowest extensions suggests that it may be important at a later stage of vessel development, perhaps as cord cells spread and form tubes. Nicosia *et al* [34] found that when rat aortas were embedded in collagen gels, the length of outgrowing microvessels increased with increasing doses of fibronectin, but the number of vessels did not increase. This implies that fibronectin does not stimulate the initial outgrowth of new vessels.

We used several criteria to identify blood vessel sprouts. Sprouts were initially recognized as tapering extensions from PECAM- or vWF-positive vessels. To screen out cutting artifacts, we considered only those extensions that arose in the middle of the confocal section series as sprouts. Because tissues were fixed before sectioning, it is unlikely that membranes vesiculated or solubilized. Nerves were initially identified by their reaction with antibodies to the 200-kDa neurofilament protein and subsequently by their shape and location. Nerves in the papillary dermis are generally thinner than blood vessels and extend for long distances without tapering. Because nerves may also wrap around blood vessels, we carefully examined confocal sections to ensure that the "sprout" extensions arose continuously from the full body of the vessel, rather than from a thin fiber running alongside. Extensions from distinctly bipolar or multiarmed cells were likewise discounted as potential sprouts, because they were likely to be pericytes or dermal cells. Remodeling of the neonatal circulation entails both growth and regression of blood vessels. Without a marker for regression, it is impossible to be certain whether a vessel is growing or regressing. Despite careful elimination of alternative possibilities, we recognize that what we present is the strong suggestion of blood vessel sprouts. Without a definitive chemical marker of sprouts, proof of the location of various integrins on vessel sprouts awaits an immunoelectronmicroscopic study.

What we have described is a static vision of the vessel sprouts. Although α6 and β4 were consistently the most intensely staining integrins at the ends of sprouts, this does not diminish the importance of other integrins, which may be present in low concentration and/or appear transiently at critical moments of angiogenesis. The integrin concentration needed to respond to the original angiogenic stimuli, and perhaps to recruit other integrins, may be much smaller than that needed to anchor the sprout physically. Whether α6 and β4 provide both signals and adhesiveness has yet to be determined.

Note Added in Proof: Recently it has been reported that αvβ3 integrin is required for angiogenesis in the chick chorioallantoic membrane (Brooks PC, Clark RA, Cheresh DA: Requirement of vascular integrin alpha v beta 3 for angiogenesis. *Science* 264:569–571, 1994).

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